Specificity of an Antibody Directed against d-Methamphetamine. Studies with Rigid and Nonrigid Analogs†,[1a]

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The specificity of an antibody directed against d-(S)-methamphetamine (MA) was determined by competitive binding assay with more than 50 compounds——metabolites, homologs, and analogs of amphetamine. The antibody appears to be specific both for the side chain and the aromatic ring of d-(S)-amphetamine (A). The basic requirements for a compound to be bound to the antibody are (a) an aromatic ring, (b) a basic nitrogen, and (c) a two-carbon chain between the aromatic ring and the nitrogen. A transoid conformation for the phenethylamine skeleton is preferred. The interaction of the antibody with compounds differing from MA or A in side-chain substitutions was directly proportional to the closeness of their structure to MA and/or A. The antibody exhibited greatly reduced affinity for ring-substituted analogs of A; the p-hydroxy metabolite of A did not bind to the antibody. A radioimmunoassay of A is described; it was utilized to study the disposition of A in dogs.

d-Amphetamine is a potent sympathomimetic amine widely abused for its central stimulant effects. It is used clinically in the treatment of hyperkinetic children,[2-4] obesity,[3,5] narcolepsy,[6] parkinsonism,[7] schizophrenia,[8] and diabetic neuropathy.[9] d-Amphetamine also produces a psychosis that has been a useful model for the study of schizophrenia.[10,11]

A simple, sensitive, and rapid method of measuring amphetamine is needed to (a) diagnose individuals who abuse the drug,[12] (b) identify the drug in cases of overdosing or poisoning, and (c) monitor plasma levels in clinical situations. Present methods involve thin-layer chromatography,[13] gas chromatography,[14] ultraviolet spectroscopy,[15] spectrophotofluorometry,[16,17] and enzymatic assay.[18] All these procedures require elaborate sample preparation and closely related compounds present in biological fluids may interfere with the assay.

Immunochemical analysis (based on the competition between radiolabeled and unlabeled antigen for binding to a limited number of sites on the specific antibody) has been used to quantitate pharmacologically important compounds at the picogram and nanogram levels, often in unprocessed serum and other biological materials.[19] Recently Cheng et al.[20] developed an antibody to d-(S)-methamphetamine, based on immunization of rabbits with a conjugate of N-(4-aminobutyl)methamphetamine coupled to bovine serum albumin.

Our interest in the development of specific antibodies to biogenic amines[21] and catecholamines stimulated us to investigate the structural and stereochemical requirements of the antibody against d-methamphetamine. As a step toward characterization of the antibody, one approach is to determine the stereochemical requirements for the hapten. This approach could help to ascertain the distances between the active groups of the antibody, to predict some details of the antibody surface, and to be of value in explaining the differences in the interaction of closely related substances.

In order to carry out this investigation, a series of analogs, homologs, and metabolites of amphetamine (Tables I–III) was evaluated for their binding affinity to the antibody. Furthermore, experiments were carried out to demonstrate the applicability of the methamphetamine antibody to measure amphetamine (1) by radioimmunoassay. The studies involved investigation of the disposition of 1 in dogs.

† This paper is dedicated to the memory of Professor Edward E. Smissman.

Results

Sensitivity of the Assay. The percent inhibition of binding of d-amphetamine-^3H by anti-methamphetamine serum in the presence of various amounts of 1 under standard conditions is shown in Figure 1; for 50% inhibition (I_{50}), 3.2 ng of 1 was required. The standard curves were linear up to 10 ng. Replicate analysis of the known samples ($n \geq 20$) demonstrated accuracy within ±2% error for each value. As little as 0.5 ng of 1 could be detected by the procedure. Blank dog plasma, urine, brain, and liver homogenates (20% w/v) did not interfere with the assay, indicating the absence of endogenous cross-reacting substances in these biological materials.

Specificity of the Antibody. The specificity of the antibody directed toward methamphetamine (3) was evaluated with several metabolites and analogs of 1 and 3; the results are shown in Tables I-III. A total of 53 compounds was tested. Of these, only seven (most closely related to the hapten; only four with pharmacological importance) were capable of causing a 50% inhibition of binding of amphetamine-^3H to the antibody, at concentrations less than tenfold that required by 1. More than half of the compounds failed to interact with the antibody ($I_{50} > 500$ ng). The remaining compounds exhibited intermediate affinity. The removal or addition of one carbon in the side chain of 1 and 3 markedly diminished the affinity.

Disposition and Distribution of Amphetamine in Dogs. Radioimmunoassay was used to measure 1 in plasma and red blood cells at various time intervals after administration of d-amphetamine (1) to dogs. The data revealed a biphasic decline in plasma levels of 1 (Figure 2). In the first 24 hr, plasma 1 levels declined initially with a $t_{1/2}$ of 5 hr (α phase) which was followed by β phase with a $t_{1/2}$ of 28 hr. Even after 48 hr, plasma 1 levels were about 1 ng/ml. Blank plasma and red blood cells had values <0.1 ng/ml of 1. The distribution of 1 was determined in selected tissues of dogs given 1 mg/kg of the drug iv. Both at 1 and 24 hr after administration of the drug, 1 was found to be concentrated in all tissues studied except muscle. At 24 hr, the tissue to plasma concentration ratio of 1 was greater than 1 (Table IV). The red cells did not contain any appreciable amount of 1.

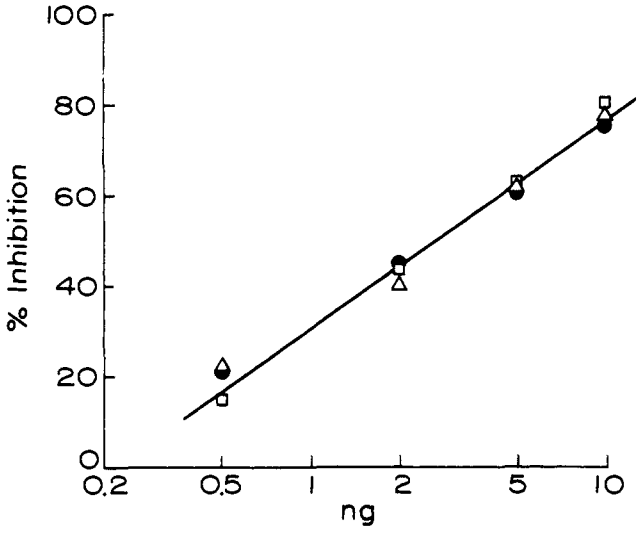


Figure 1. Standard curve for the inhibition of binding of *d*-amphetamine-^3H by an antibody directed against *d*-methamphetamine by *d*-amphetamine: □, in 0.01 *M* phosphate buffered saline; ●, in dog urine, diluted 1:1 with buffer; △, in dog plasma. Each point represents the average of three determinations.

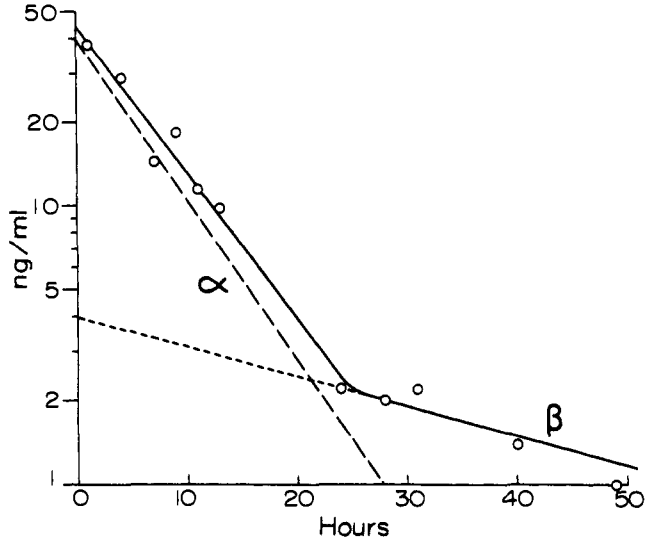


Figure 2. Plasma levels of *d*-amphetamine after iv administration of 0.5 mg/kg of *d*-amphetamine sulfate. The $t_{1/2}$'s for α and β phases (dashed lines) were 5 and 28 hr, respectively. Each point represents the average for four animals. The range of concentrations (ng/ml) for 1, 4, 7, 9, 11, 13, 24, 28, 31, 40, and 48 hr was 36-42, 28-30, 15-18, 19-21, 12-15, 10-11, 2.2-2.5, 2-2.3, 1.8-2.1, 1.5-1.8, and 1-1.2, respectively, for the levels of 1 in dog plasma as determined by radioimmunoassay.

Discussion

The classical studies of Landsteiner and his associates[22] demonstrated that low-molecular-weight compounds that per se are incapable of evoking antibody formation could be covalently linked to carrier proteins which then act as immunogenic agents. The immune response could then be directed against such low-molecular-weight substances (e.g., drugs). The specificity of the resulting antiserum is influenced by various factors such as the size of the bridge between hapten and carrier protein, degrees of freedom, and the site of conjugation on the hapten molecule.[23-25]

The methamphetamine antibody used in this study was produced by immunization of rabbits with an antigen formed by coupling *d*-*N*-(4-aminobutyl)methamphetamine to bovine serum albumin.[20] For this antigen, the bridge between the protein and amphetamine moiety consists of four carbons and one nitrogen (4 methylene carbons and nitrogen of amide bond). The antibody formed in response to this antigen was predicted to have high affinity and specificity for *d*-methamphetamine (**3**) and *d*-amphetamine (**1**). Immunization of rabbits with a conjugate formed by direct coupling of **1** to bovine serum albumin resulted in the formation of antibodies with lower affinity and specificity to **1**.[20] The above findings are in agreement with those of Midgeley and Niswender[26] that the greatest specificity of the antibody for the hapten molecule is for an area farthest away from the site of protein attachment.

Amphetamine contains two distinct regions, the side chain and phenyl ring, in which structural changes influence interactions with the antibody. The inhibition data (Tables I–III) indicate that the specificity of the antiserum is directed toward both the side chain and the aromatic ring of the molecule, with more specificity for the latter.

The interaction of the antibody with compounds differing from **1** in side-chain substitutions was directly proportional to the closeness of their structure to the hapten. The specificity was so pronounced that the antibody could readily distinguish the removal or addition of one carbon, relocation of a carbon, or introduction of a heteroatom in the side chain. Thus, removal of the β-carbon (**16**) or the α-substituent (**15**) as well as introduction of additional carbon (**17**) or oxygen (**18**) nearly completely abolished the affinity of the antibody for the substrate. Substitution of α-methyl by hydroxymethyl (**20**) or cyclohexyl (**11**) resulted in compounds with decreased affinity for the antibody; substitution by a phenyl group (**13**) greatly reduced the binding. Translocation of substituents from the α to the β position in the side chain (**6**, **12**) or introduction of additional methyl substituents (**7–10**) or hydroxy substituents (**19**) decreased the affinity several-fold. Introduction of bulky groups on nitrogen (**14**) or carbon (**11–13, 29–31, 34, 36, 37, 39, 42–45**) resulted in either abolishment or greatly attenuated affinity. The presence of bulky groups apparently interferes with the "fit" necessary for binding to the surface of the antibody.

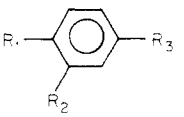
Acylation of nitrogen (**4**, **5**) completely abolished the affinity. Thus, a basic nitrogen on the substrate molecule is necessary for binding to this antibody.

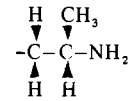
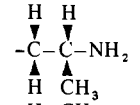
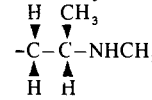
Substitution of the aromatic ring by a cyclohexyl ring, such as in compounds **40** and **41**, diminished the affinity considerably. From these data it appears that the steric geometry and the electronic distribution of the aromatic ring are critical haptenic determinants, probably essential for orienting the remainder of the molecule onto the antibody surface.

It is noteworthy that the antibody displayed some degree of selectivity toward optical isomers. It was found that *l*-amphetamine (**2**) is about one-fourth as effective as *d*-amphetamine (**1**) in inhibiting the antigen–antibody interaction. Interestingly, these data parallel the observation that in the corpus striatum, *d*-amphetamine appeared to be approximately 4–5 times more potent than *l*-amphetamine in inhibiting the uptake of norepinephrine.[27,28]

Analogs with substitutions on the aromatic ring (**21–27**) resulted in markedly decreased affinity except in the case of *p*-chloroamphetamine (**22**) which still retained some binding. The introduction of a hydroxy group at the para position of the ring (**21**) completely abolished the affinity of this substrate for the antibody. Thus, this pharmacologically potent metabolite of **1** will not interfere with the radioimmunoassay of amphetamine. In an analogous case, the *p*-hydroxy metabolite of diphenylhydantoin was found to have a much lower affinity than the parent drug for an antibody directed against diphenylhydantoin.[29]

Table I. Inhibition of Binding of Amphetamine-^3H by Anti-Methamphetamine Serum by Ring and Side-Chain Substituted Phenethylamine Analogs and Homologs



	Compound	R_1	R_2	R_3	I_{50}, ng
1	*d*-(*S*)-Amphetamine	H	H		3.2
2	*l*-(*R*)-Amphetamine	H	H		8.8
3	*d*-(*S*)-Methamphetamine	H	H		2.7
4	*N*-Acetyl-*l*-amphetamine	H	H	$-CH_2CH(CH_3)NHCOCH_3$	>500
5	*N*-Trifluoroacetyl-*l*-amphetamine	H	H	$-CH_2CH(CH_3)NHCOCF_3$	>500
6	β-Methylphenethylamine	H	H	$-CH(CH_3)CH_2NH_2$	34
7	*erythro*-2-Amino-3-phenylbutane	H	H	$-CH(CH_3)CH(CH_3)NH_2$	24
8	*threo*-2-Amino-3-phenylbutane	H	H	$-CH(CH_3)CH(CH_3)NH_2$	33
9	α,α-Dimethylphenethylamine (phentermine)	H	H	$-CH_2C[(CH_3)_2]NH_2$	95
10	*N*,α,α-Trimethylphenethylamine (mephentermine)	H	H	$-CH_2C[(CH_3)_2]NHCH_3$	12
11	α-Cyclohexyl-*N*-methylphenethylamine	H	H	$-CH_2CH(c\text{-}C_6H_{11})NHCH_3$	31
12	β-Cyclohexyl-*N*-methylphenethylamine	H	H	$-CH(c\text{-}C_6H_{11})CH_2NHCH_3$	>500
13	1,2-Diphenylethylamine	H	H	$-CH_2CH(C_6H_5)NH_2$	>500
14	*erythro*-2-Isopropylamino-3-phenylbutane	H	H	$-CH(CH_3)CH(CH_3)NHCH(CH_3)_2$	160
15	Phenethylamine	H	H	$-CH_2CH_2NH_2$	>500
16	*d*-Methylbenzylamine	H	H	$-CH(CH_3)NH_2$	>500
17	1-Methyl-3-phenylpropylamine	H	H	$-CH_2CH_2CH(CH_3)NH_2$	>500
18	1-Methyl-2-phenoxyethylamine	H	H	$-OCH_2CH(CH_3)NH_2$	>500
19	α-(1-Aminoethyl)benzyl alcohol (norephedrine)	H	H	$-CH(OH)CH_2(CH_3)NH_2$	20
20	3-Phenyl-2-aminopropanol	H	H	$-CH_2CH(CH_2OH)NH_2$	85
21	*dl*-*p*-Hydroxyamphetamine	OH	H	$-CH_2CH(CH_3)NH_2$	>500
22	*dl*-*p*-Chloroamphetamine	Cl	H	$-CH_2CH(CH_3)NH_2$	70
23	*p*-Chloro-α,α-dimethylphenethylamine (chlorophentermine)	Cl	H	$-CH_2C[(CH_3)_2]NH_2$	>500
24	Tyramine	OH	H	$-CH_2CH_2NH_2$	>500
25	Dopamine	OH	OH	$-CH_2CH_2NH_2$	>500
26	*l*-Norepinephrine	OH	OH	$-CH(OH)CH_2NH_2$	>500
27	*l*-Epinephrine	OH	OH	$-CH(OH)CH_2NHCH_3$	>500

Similarly, dopamine exhibited very low affinity for the anti-tyramine serum[21] and the hydroxy metabolites of probenecid did not interact with the antibody produced in response to probenecid containing antigen.[30] Smith et al.[31] also observed that digitoxin had a much lower affinity than digoxin for an antibody prepared against a digoxin containing conjugate.

In order to investigate the possibility that **1** exists in a preferred conformation when bound to the antibody, several analogs of **1** were tested in which the phenylethylamine nucleus was incorporated into rigid and semirigid systems (Tables II and III). Among these compounds, only those isomers which have the amino and the phenyl group in the trans conformation such as *trans*-phenylcyclopropylamine (**28**) or molecules which could easily attain this conformation such as **29**, **30**, **35–37**, and **39** demonstrated affinity to the methamphetamine antibody. In the semirigid system, β-methylamphetamine, the erythro form (**7**), was bound somewhat more effectively to the antibody than its threo isomer (**8**). Because of the bulky methyl groups, the lower energy transoid conformation (phenyl ring with respect to nitrogen) would be favored for the erythro form; the preferred conformation for the threo form will be the higher energy "gauche". In the case of *dl*-2,3-dimethylindoline (**32**) in which the amino group and the phenyl ring are in a fixed cisoid geometry, the compound failed to bind to the antibody. Furthermore, among the benzobicyclo[2.2.2]octene analogs of **1**, only the exo isomers **42** and **44** (Table III) produced some inhibition of the binding of tritiated amphetamine to the antibody, while the endo compounds **43** and **45** were devoid of this property.

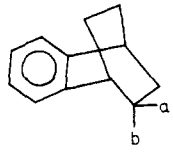
As an additional test for specificity of the radioimmunoassay, we carried out limited studies of the dispo-

Table II. Inhibition of the Binding of Amphetamine-^3H by Anti-Methamphetamine Serum by Rigid[a] and Semirigid Analogs of Amphetamine

	Compound	I_{50}, ng
28	*trans*-2-Phenylcyclopropylamine	37
29	*N,N*-Dimethyl-1,2,3,4-tetrahydronaphthylamine	95
30	3-Methyl-2-phenylmorpholine (phenmetrazine)	61
31	*dl-threo*-2-Phenyl-2-piperidineacetic acid methyl ester (methyl phenidate)	>500
32	*dl*-2,3-Dimethylindoline	>500
33	*cis*-1-Amino-2-phenylcyclohexane	>500
34	*trans*-1-Isopropylamino-2-phenylcyclohexane	>500
35	3-Phenyldecahydroquinoline	5.5
36	6-Amino-6,7,8,9-tetrahydro-5*H*-benzocycloheptane	70
37	*trans*-5-Methyl-1,2,3,4,4a,5,6,10b-octahydro[*f*]quinoline	60
38	2-Benzylpyridine	>500
39	2-Benzylpiperidine	70
40	2-Cyclohexylmethylpiperidine	>500
41	*N*,α-Dimethylcyclohexane ethylamine (propylhexedrine)	>500

[a] Eight *trans*-decalin[42] analogs had I_{50} values greater than 500 ng.

Table III. Inhibition of the Binding of Amphetamine-^3H by Anti-Methamphetamine Serum by Phenethylamine-Like Compounds Made Rigid by Incorporating Them into a Benzobicyclo[2.2.2]octene System[a]



	Compound	a	b	I_{50}, ng
42	2-Aminobenzobicyclo[2.2.2]octene (exo)	NH_2	H	200
43	2-Aminobenzobicyclo[2.2.2]octene (endo)	H	NH_2	>500
44	2-*N*-Methylaminobenzobicyclo[2.2.2]octene (exo)	$NHCH_3$	H	100
45	2-*N*-Methylaminobenzobicyclo[2.2.2]octene (endo)	H	$NHCH_3$	>500

[a] Compounds 42–45 were synthesized by Grunewald et al.[37]

sition of amphetamine in dogs at doses comparable to those used therapeutically in man. We found that the procedure was specific; endogenous substances present in biological fluids (plasma, blood, urine, and tissue homogenates etc.) or the *p*-hydroxy metabolite of amphetamine (21) did not interfere with the assay. The sensitivity of the method allowed us to follow the disappearance of amphetamine from plasma of dogs over a period of 48 hr after the dose. The results of our pharmacokinetic studies are in agreement with previous data.[32-34] Decline of plasma concentration exhibited a biphasic decay. Plasma 1 level data and extensive localization in tissues even after 24 hr following drug administration suggest the presence of a deep compartment for this compound.

Tuomisto et al.[35,36] showed that conformational changes in the amphetamine molecule resulted in analogs with different inhibitory activity for the uptake of biogenic amines by rabbit platelets. Preliminary studies by Grunewald et al.[37] also demonstrated that a preferred transoid conformation for 1 is needed for the release of dopamine from corpus striatum and norepinephrine from the cortex. Based upon these observations and that of Ferris et al.[27] and others,[28,38] it is conceivable that the methamphetamine (amphetamine) antibody represents a potential model for an amphetamine receptor site. Antibodies directed against steroids and morphine have been reported to interfere with the physiological actions of the steroids[39] and morphine.[40] It will be worthwhile to study whether antibodies directed against amphetamine interfere with the pharmacological and behavioral effects of amphetamine.

Experimental Section

The synthesis of compounds **7**, **8**, **12**, **14**, **33–35**, and **37** (Tables I and II) has been described.[41-43] Compounds **2**, **3**, **6**, 11, **13**, **15–20**, **28**, **29**, **32**, and **38** were obtained from Aldrich Chemical Co. Compounds **22**, **24**, **26**, **27**, and bovine serum albumin were purchased from Sigma Chemical Co. Compounds **9**, **10**, **23**, **25**, **30**, and **41** were obtained from commercial pharmaceutical tablets; the active ingredients were extracted and purified and the purity was checked by TLC and ir (Beckman Acculab 4). Compounds 1 and **21** were supplied by Smith Kline & French Laboratories. Compound **31** was a gift from Ciba-Geigy. Compound **36** was a gift from Dr. N. Anand[44] (Central Drug Research Institute, Lucknow, India) and **42–45** (Table III) were gifts from Dr. G. Grunewald[37] (Department of Medicinal Chemistry, University of Kansas, Lawrence, Kan.). Compounds 4 and **5** were prepared by conventional acylation of **2**. The antibody (serum from one rabbit, diluted 1:10 with phosphate buffer) against *d*-methamphetamine (prepared by the procedure of Cheng et al.[20]) was obtained from ICN Laboratories. *d*-Amphetamine-^3H (specific activity 9 Ci/mmol) was obtained from New England Nuclear. Radioactivity was measured in a liquid scintillation counter (Beckman LS-133) using a scintillation fluid prepared from 7 g of PPO, 0.36 g of POPOP, 200 ml of Beckman Biosolv-BBS-3, and 1 l. of toluene[21] (counting efficiency = 40% for ^3H). NMR spectra were recorded on a Varian EM-360 60-MHz NMR spectrometer. Centrifugation was performed on an International B-20 refrigerated centrifuge.

***dl*-2-Benzylpiperidine Hydrochloride (39).** 2-Benzylpyridine (**38**, 1 g, 6 mmol) was dissolved in absolute EtOH (25 ml) containing 1 ml of concentrated HCl and reduced over PtO_2 (100 mg)

Table IV. Tissue Distribution of *d*-Amphetamine in Dogs

Tissue	Amphetamine levels,[a] ng/g		Tissue/plasma concn ratio[a,b]	
	1 hr	24 hr	1 hr	24 hr
Lung	1648.7 (1600-1675)	360.7 (310-400)	6.33 (6.15-6.44)	11.63 (11.29-12.90)
Muscle	117.2 (110-124)	73.7 (60-85)	0.44 (0.42-0.47)	2.37 (1.93-2.74)
Spinal cord	1061.5 (1015-1111)	70.5 (57-85)	4.08 (3.90-4.27)	2.26 (1.83-2.74)
Kidney	328.7 (300-360)	399.0 (350-450)	1.26 (1.15-1.38)	12.46 (9.67-14.51)
Adrenals	1514.0 (1486-1560)	137.7 (130-148)	5.82 (5.71-6.00)	4.44 (4.19-4.77)
Spleen	1927.7 (1900-1960)	372.5 (325-410)	7.40 (7.30-7.53)	12.01 (10.48-13.22)
Brain	1243.7 (1200-1310)	207.0 (198-215)	4.77 (4.61-5.03)	6.65 (6.3-6.93)
Liver	1277.5 (1265-1295)	192.5 (170-210)	4.91 (4.86-4.98)	6.20 (5.48-6.77)
Heart	1154.0 (1149-1160)	83.0 (90-96)	4.43 (4.42-4.46)	2.67 (2.61-3.10)
Spinal fluid	442.5 (400-485)	53.2 (48-60)	1.69 (1.53-1.86)	1.71 (1.54-1.93)

[a] Average; numbers in parentheses represent the range for four dogs. [b] Average concentrations of 1 in plasma at time of sacrifice were 1 hr, 260 ng/ml, 24 hr, 31 ng/ml (the range was <10% for each value); for red blood cells (lysate) at 7 hr after dose the value was <1 ng/ml.

at an initial H_2 pressure of 1.5 kg/cm^2 at room temperature. After 2 hr the mixture was filtered and the filtrate was concentrated in vacuo at 45°. The residue was recrystallized from EtOH–*n*-heptane: mp 125–130° (lit.[45] 137°); NMR (D_2O) δ 7.50 (s, 5, aromatic), 3.4–2.65 and 2.0–0.9 (m, 11 H).

***dl*-2-Cyclohexylmethylpiperidine Hydrochloride (40).** 2-Benzylpyridine (**38**, 1 g, 6 mmol) was reduced as above over PtO_2 (350 mg) with H_2 pressure of 2.0 kg/cm^2 for 24 hr. The product was recrystallized from *i*-PrOH: mp 206–209° (lit.[46] 209–210°); NMR (D_2O) δ 3.7–2.8 and 2.3–0.7 (complex, aliphatic).

Radioimmunoassay Procedure. The method used was similar to that described earlier.[21] Into 12 × 75 mm plastic tubes (Lab Tek culture tube, Scientific Products, Inc.) were placed 0.6 ml of a solution of 0.2% bovine serum albumin w/v in phosphate buffer (pH 7.6, 0.01 *M* Na_2HPO_4 containing 0.15 *M* NaCl and 0.1% NaN_3 w/v), then 0.1 ml of 0.1 *M* Na_2 EDTA (pH 7.6), 0.1 ml of antibody solution (1:200 dilution), 0.15 ng of *d*-amphetamine-^3H (8000 cpm) in 0.1 ml of buffered saline, and unlabeled 1 (0.1–20 ng for a standard curve) or unknown samples (0.1 ml of a solution of test compounds or 0.1 ml of plasma, tissue homogenate, or diluted urine was added). The tubes were capped and incubated at 0–4° for 24 hr. After incubation the free and antibody-bound 1 was separated by ultrafiltration. Dialysis tubing (U-shaped, cellulose 0.25 in., size 8, VWR Scientific, Inc) was placed in the culture tube in such a way that about 0.25 in. protruded out on each side. Essentially all of the incubation solution was transferred into the dialysis tubing. The tubes were capped and centrifuged at 2000g at 4° for 24 hr; 0.15–0.2 ml of ultrafiltrate was formed. Aliquots (0.1 ml) of both inside and outside phases were dissolved in 12 ml of scintillation fluid and counted.

Each control and unknown sample was assayed in triplicate. All samples were counted to ±2% error. Standard curves were also obtained by adding the same amount of amphetamine (0.1–20 ng) to 50% urine, plasma, 20% brain, and liver homogenates.

Calculation of Binding of Amphetamine-^3H to Anti-Methamphetamine Serum. To obtain the extent of binding with high accuracy, the concentration of amphetamine-^3H both in the inside and outside phases was measured independently. The present binding was then calculated from the formula: % = $[(C_i - C_o)/C_i] \times 100$. C_i = concentration of amphetamine-^3H (free + antibody-bound) inside the dialysis bag, C_o = concentration of amphetamine-^3H (free) in the outside phase (buffer). Possible decomposition of the labeled amphetamine during incubation was evaluated as follows. An aliquot of the outside phase was applied directly to silica gel G (Merck) 0.25-mm thin-layer plates. A freshly prepared solution of 1 was also applied to the plate as reference. The plates were developed in the following solvent systems: (a) EtOAc–MeOH–H_2O (80:15:5 v/v) and (b) MeOH–NH_4OH (100:1.5 v/v). Sections (1 cm^2) of the gel were scraped into counting vials and scintillation fluid was added. Radioactivity was measured after mixing and allowing the samples to stand for 2 hr. No decomposition was found since almost all of the radioactivity was contained in the spot corresponding to 1 (R_f in systems a and b = 0.29 and 0.47, respectively).

Specificity of the Antibody. Most of the compounds (1 mg) were dissolved in phosphate-buffered saline (pH 7.6) to give a concentration of 10 μg/ml. From this stock solution, standards were prepared (5–500 ng/0.1 ml), which were used for the competitive assay. Compounds poorly soluble in water were first dissolved in a minimum amount of MeOH and then diluted with buffer.

Specificity of radioimmunoassay was determined by measuring the displacement of amphetamine-^3H from the amphetamine-^3H–antibody complex by known quantities of the inhibitor. The percent inhibition of binding of amphetamine-^3H to the antibody was calculated by determining the percent of counts displaced from the amphetamine-^3H–antibody complex by a certain amount of the inhibitor.

Measurement of Amphetamine Concentration in Plasma, Urine, and Tissues of Dogs after Administration of *d*-Amphetamine Sulfate. Dogs (four females, 15–20 kg) were injected iv with *d*-amphetamine sulfate (0.5 mg/kg as free base) in 3 ml of normal saline. The dogs were placed in metabolic cages and were maintained on Purina dog chow and water ad libitum. Blood samples (heparinized) and urine were collected at various time intervals up to 48 hr; plasma was separated by centrifugation (500 g, 20 min, 20°). All biological samples were stored at −10° prior to assay (within 24 hr). In order to determine the tissue levels of 1, eight dogs (12–15 kg) were injected iv with *d*-amphetamine sulfate (1 mg/kg as free base). Four dogs were sacrificed at 1 hr and the others at 24 hr by an overdose of sodium pentobarbital. Selected tissues were removed, weighed, cooled (0°), and then homogenized (20% w/v) in 0.1 *N* HCl. The homogenates were centrifuged at 10000g for 20 min at 4°. The supernatant was removed and stored at −10° until analyzed by radioimmunoassay (within 24 hr). The recovery of added 1 to tissue homogenates was >95%.

For samples in which the levels of 1 were too low to measure by a direct method, 1 was extracted from biological fluids (plasma, bile, cerebrospinal fluid, and tissue homogenates) as follows. An aliquot of biological material (usually 1 ml, up to 5 ml) was placed in a glass-stoppered centrifuge tube; 1 ml of 0.1 *N* NaOH and 20 ml of EtOAc were added. The mixture was shaken for 30 min and then centrifuged. An aliquot of the organic phase was removed and evaporated to dryness; the residue was reconstituted in buffered saline and assayed by radioimmunoassay as described above. The recovery of added 1 by this extraction procedure was >90%.

Amphetamine was measured in red blood cells as follows. Cells (1–3 ml) were lysed by incubating for 1 hr at 37° with an equal volume of H_2O. The lysate was centrifuged at 500g and an aliquot of the supernatant was extracted with EtOAc and analyzed for 1 as described above.

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Aporphines. 14.[1] Dopaminergic and Antinociceptive Activity of Aporphine Derivatives. Synthesis of 10-Hydroxyaporphines and 10-Hydroxy-*N*-*n*-propylnoraporphine[†,2]

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The synthesis of racemic 10-hydroxyaporphine [(±)-**2a**] and 10-hydroxy-*N*-*n*-propylnoraporphine [(±)-**2b**] is described. The method involved a Reissert alkylation–Pschorr cyclization route. The dopaminergic activity of (±)-**2b** was evaluated in comparison with L-Dopa, (–)-apomorphine (**1a**), (±)-*N*-*n*-propylnorapomorphine (NPA) (**1b**), and (±)-11-hydroxy-*N*-*n*-propylnoraporphine [(±)-11-OH-PNA] by the behavioral model of rotational behavior in animals after unilateral lesion of the ascending DA pathways. The dopaminergic activity of NPA and 11-OH-PNA is essentially equivalent to L-Dopa and (–)-apomorphine, and both are more active than (±)-**2b**. Furthermore, (±)-NPA (threshold dose, 5 μg/kg) appears to be even more potent than (–)-apomorphine (threshold dose, 25 μg/kg). The duration of action of NPA and 11-OH-PNA is considerably longer than that obtained with L-Dopa. The antinociceptive activity of (±)-**2b** was evaluated by the tail-flick procedure and compared with **1a**, **2b**, morphine, and L-Dopa. Weak but significant antinociceptive activity was shown by (±)-**2b** and by (±)-**1b** but not by (–)-apomorphine. This effect was not antagonized by naloxone. The finding that (±)-**2b** and particularly (±)-11-OH-PNA are active in doses from 500 to 50 μg/kg, respectively, in causing rotational behavior further supports previous studies indicating that *N*-*n*-propyl derivatives of monohydroxylated aporphines were more active than the corresponding parent *N*-methyl derivatives as DA receptor agonists and that a catechol system is not an absolute requirement for dopaminergic activity in such aporphines.

In continuing our studies involving the design of dopamine agonists related to apomorphine (**1a**), structures which can be considered as conformationally rigid forms of dopamine, we wished to synthesize and evaluate the monohydroxyaporphines, 10-hydroxyaporphine **2a**, and its *N*-propyl homolog (10-OH-PNA) **2b**.

In our previous studies involving functionally substituted aporphines we indicated that dopaminergic activity can reside in monohydroxyaporphines substituted in the 11 position and that the *N*-*n*-propyl derivatives of the hydroxylated aporphines were more active than the corresponding parent compounds. The evidence that such monohydroxyaporphines were direct-acting dopamine agonists was supported by the behavioral model of rotational behavior in animals after unilateral lesion of the nigrostriatal pathway[3a] and the ability of such compounds to produce stereotyped behavioral syndrome in the rat similar to apomorphine.[3b] These studies[1,3b] confirmed that (–)-*N*-*n*-propylnorapomorphine [(–)-NPA] (**1b**) first described by Koch et al.[4] was considerably more potent than (–)-apomorphine [(–)-APO] (**1a**) and that differences in stereotypic activity of APO and NPA may be related to

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